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Plant Regeneration of Pummelo cv. Cikoneng from Cotyledon and Epicotyl

Iswari Saraswati Dewi[1], I. H. Rahman[2], and Bambang Sapta Purwoko[2*]

[1]Indonesian Center of Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD)
Jl. Tentara Pelajar 3A, Bogor 16114, Indonesia
[2]Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University
Jl. Meranti, Darmaga, Bogor 16680, Indonesia

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ABSTRACT

In vitro conservation needs highly efficient micropropagation protocol. The objective of the research was to obtain an efficient and reproducible protocol for pummelo (Citrus maxima (Burm.) Merr.) micropropagation through direct shoot formation. The experiment was arranged in a completely randomized design with two factors and 20 replications. The 1st factor was type of explant, i.e. cotyledon and epicotyl segments of Pummelo cv. Cikoneng, while the 2nd factor was the media composition as follow (1) MS + 1.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; (2) MS + 2.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; (3) MS + 1.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; (4) MS + 2.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 1.0 mg NAA L^{-1}; (5) MS + 2.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 1.0 mg NAA L^{-1}. Observation was conducted on days to shoot induction, number of explant forming shoots, shoot height, number of shoots, leaves, and roots. The results showed that adventitious shoots emerged from callus in epicotyl (6-8 WAP), but adventitious shoots could emerge directly without an intervening callus phase from cotyledon (4-5 WAP). Shoots emerged from epicotyl were weak and vitrous due to hyperhydricity, thus they can not be used for micropropagation. Cotyledons cultured in media MS + 1.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1} or media MS + 1.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1} gave the highest percentage of explant forming adventitious shoot (38.8 and 26.3%), highest efficiency of shoot formation (62.5 dan 72.5%), and highest numbers of leaves (value of 1.9 leaves $shoot^{-1}$) and roots (1.1 roots $shoot^{-1}$) compared to other media. Since shoot height and number of leaves and root were not significantly different in both media, thus cotyledon and media MS + 1.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1}+ 0.5 mg NAA L^{-1} which was less in Kinetin is suggested to be used for pummelo micropropagation.

Keywords: Citrus maxima, epicotyl, cotyledon, regeneration, pummelo

ABSTRAK

Konservasi in vitro memerlukan protokol mikropropagasi yang efisien. Penelitian ini dilakukan untuk mendapatkan protokol mikropropagasi pamelo (Citrus maxima (Burm.) Merr.) yang optimum melalui pembentukan tunas langsung. Percobaan disusun berdasarkan racangan acak lengkap dua faktor dengan 20 ulangan. Faktor pertama adalah jenis eksplan, yaitu potongan kotiledon dan epikotil Pamelo cv. Cikoneng, sedangkan faktor kedua adalah komposisi media yaitu 1) MS + 1.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; (2) MS + 2.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; (3) MS + 1.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; (4) MS +2.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 1.0 mg NAA L^{-1}; (5) MS + 2.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 1.0 mg NAA L^{-1}. Pengamatan dilakukan terhadap waktu inisiasi tunas, jumlah eksplan yang membentuk tunas, tinggi tunas, jumlah tunas, daun dan akar. Hasil menunjukkan bahwa tunas adventif pada epikotil didahului oleh pembentukan kalus (6-8 MSK), sedangkan pada kotiledon langsung terbentuk (4-5 MSK). Tunas dari epikotil tampak lemah dan transparan sehingga tidak dapat digunakan untuk perbanyakan pamelo secara in vitro. Kombinasi perlakuan kotiledon dan media MS + 1.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1} atau media MS + 1.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1} menghasilkan persentase eksplan bertunas (38.8 dan 26.3%), efisiensi pembentukan tunas (62.5 dan 72.5%), jumlah daun (1.9 daun $tunas^{-1}$) dan akar (1.1 akar $tunas^{-1}$) yang lebih tinggi dibandingkan perlakuan media lain. Tinggi tunas serta jumlah daun dan akar yang dihasilkan kotiledon pada kedua media terbaik tidak berbeda, sehingga penggunaan kotiledon pada media MS + 1.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1} yang mempunyai lebih sedikit kinetin dianjurkan untuk mikropropagasi pamelo.

Kata kunci: Citrus maxima, epikotil, kotiledon, pamelo, regenerasi

* Corresponding author. e-mail: bambangpurwoko@gmail.com

INTRODUCTION

Plant genetic resources are rapidly vanishing due to deprivation of habitat or selective reduction, leading to genetic vulnerability endangering the plant industry and future needs. Germplasm conservation can cover a range of work including exploration, collection, characterization, maintenance, and rejuvenation of particular group of both cultivated and wild relative of plants. It is a very important basis of breeding material for specific objective. Therefore, germplasm conservation must be one of the main focuses in supporting agriculture development as well as maintaining the existence of genetic variability for future needs.

Among the fruit crops, the genus Citrus and its relatives are important (Ye *et al*., 2009). Pummelo (*Citrus maxima* (Burm.) Merr.), the largest fruit size among citrus, is an under-utilized fruit with a potential for commercialization. Pummelo originated most likely from Indonesia where it grew wild (Niyomdham, 2003; Karsinah *et al*., 2002). It is also presumed to have been grown by the Chinese for thousands of years (Min, 1997). However, most believed that its primary centre of diversity is Southeast Asia (Malaysia, Thailand, Indonesia, the Philippine) from where it spreads to China, the Indian subcontinent and to Iran (Paudyal and Haq, 2008). In Indonesia, most of the pummelo are grown in homestead gardens or farmer field and only several cultivars are conserved *ex situ* in the field gene bank such as in Botanical Garden and Research Institute for Citrus and Other Tropical Fruits (Balitjestro), Malang. Such collections are vulnerable to biotic and abiotic hazards. For example three commercial pummelos, i.e. cultivar Nambangan from Magetan, Bali Jingga a local cultivar from Pati and cultivar Cikoneng from Sumedang were considered extinct in 1980s, because of the outbreak of CVPD (Citrus Vein Phloem Degeneration) and *Botryodiplodia theobromae* (Source: Kompas, May 19, 2003). In the end of 1990s, cultivar Cikoneng could be restored from two healthy plants grown in the forest, while local cultivar Bali Jingga could be restored from the only old plant left in the garden. Therefore, there is an urgent need to seek other alternative *ex situ* conservation of the pummelo germplasm.

In vitro conservation offers alternative strategies for short, medium, and long-term storage of germplasm. Short and medium-term storage techniques require high efficient protocol in micropropagation, maximum survival rate, and reduction of subculturing frequency (Sarwar and Siddiqui, 2004). Therefore, the need for a protocol that results in efficient differentiation, shoot development and whole plant regeneration is an essential requirement to initiate mother stock for plant conservation *in vitro*, especially when the slow growth technique is selected. In that case, only direct organogenesis-derived shoot can be used as explants. Indirect organogenesis, which gave callus-derived shoots, may lead to somaclonal variation (Paudyal and Haq, 2000; Kabir *et al*., 2008).

There are a number of reports of regeneration of adventitious shoots from various explants, such as leaf of sour cherry and sweet orange (Song and Sink, 2005; Khan *et al*., 2009), epicotyl of sweet orange (Filho, 2001), stem of mature lime tree (Al-Bahrany, 2002), cotyledon-derived callus of pummelo (Begum *et al*., 2003), hypocotyl and epicotyl of blackgram (Saini and Jaiwal, 2002). Since pummelo cv. Cikoneng was self compatible, seeds and part of seedlings can be used as explants in order to have true to type seedlings (Rahayu *et al*., 2012).

Among plant hormones, cytokinins and auxins, are essential for normal plant growth and development. The activity of cytokinins is essential to maintain undifferentiated cells in shoot apical meristem (SAM) and to promote cell differentiation in the root apical meristem (RAM). Induction of adventitious shoot has been conducted in epicotyl and hypocotyl of sweet orange using N6-benzyladenin (BA) and abscisic Acid (ABA), but the shoot did not grow further after 4-6 weeks after differentiation (Maggon and Singh, 1995). However, in lime the addition of auxin such as Naphthalene acetic Acid (NAA) to medium containing BA and Kinetin, either inhibited, stimulated or did not affect shoot multiplication, depending on the concentration of cytokinins (Al-Bahrany, 2002). The objective of the present research was to obtain an efficient and reproducible protocol for pummelo micropropagation through direct shoot formation.

MATERIALS AND METHODS

This research was conducted at Cell Biology Laboratory-ICABIOGRAD. Seeds of Pummelo cv. Cikoneng were obtained from Orchard of H. Soom in Sumedang, West Java. Experiment was arranged in a completely randomized design with two factors and 20 replications. The first factor was type of explants, i.e. cotyledon and epicotyl segments (1.5-2.0 mm thickness) of Pummelo cv. Cikoneng, while the second factor was the media composition. The media composition were as follow: (1) MS+1.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1}+0.5 mg NAA L^{-1}; (2) MS + 2.0 mg BAP L^{-1}+0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; (3) MS + 1.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; (4) MS + 2.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 1.0 mg NAA L^{-1}; (5) MS + 2.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 1.0 mg NAA L^{-1}. Four cotyledons (1.0-1.5 cm) were placed abaxially and 5 epicotyl segments (1.0 cm) were placed horizontally (Begum *et al*., 2003) on the medium in each culture jar.

The culture medium was based on the inorganic salts of Murashige and Skoog (1962) supplemented with 100 mg myo-inositol L^{-1}, 10 mg thiamine-HCl L^{-1}, 10 mg pyridoxine-HCl L^{-1}, 1 mg nicotinic acid L^{-1} and 30 g sucrose L^{-1}. The pH of the medium was adjusted to 5.8 before the addition of 8 g agar L^{-1}. The mixture was then autoclaved at 18-20 Psi at 120 ^{o}C for 20 minutes. The medium was dispensed as 40 mL aliquots into culture jars. All cultures were incubated in the dark at 22$\pm$2 ^{o}C until the explants showed any formation of nodules as an indication of shoot initiation. The culture then was incubated under a 16 h day length, with an irradiance

provided by cool white fluorescent tubes at a temperature of 26±2 °C. Shoots with leaf were transferred to MS + 500 mg malt extract L^{-1} + 10 mg NAA L^{-1} +100 mg activated charcoal L^{-1} for rooting. Observation was made on number of days to induce shoot, number of explant forming shoots, number of shoots, shoot height, number of leaves, and number of roots.

RESULTS AND DISCUSSION

During shoot initiation, change of color and size occured on cotyledon of pummelo in all media (Figure 1). Each cell has different differentiation potential (Hopkins, 2004). Cotyledon enlarged and its color changed from yellow to green 1 week after planting (WAP). Previous research stated that growth of cotyledon of bottle gourd was promoted by cytokinin and later the cotyledon became photosynthetic organ and changed its color to green (Saha *et al*., 2007). In this research, adventitious shoot initiation in pummelo cotyledon *in vitro* appeared as nodules or protrution in greening cotyledons which later became shoots (Figure 1B). It was also observed some cotyledon which changed its color from yellow to pale yellow (Figure 1, D) will later changed to brown, became smaller and dead.

The time needed to form first adventitious shoots ranged between 4-5 weeks after planting (WAP) in all media. Adventitious shoots grew from cotyledon-cut side (Figure 1, upper right and bottom). Cotyledon position also influenced growth direction or shoot polarity. In pre-experiment, when inner side of the cotyledon was positioned in contact with media (adaxial position), shoot appeared toward the media and later grew upward. The earliest shoot was formed in media 1, i.e. MS + 1.0 mg BAP L^{-1}+ 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1} and media 3, i.e MS + 1.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}. It seemed that the addition of the cytokinin is considered to be an absolute requirement, but the hormone requirement for optimal shoot regeneration varies, and is considered to be genotype-specific (Singh, 2002; Usman *et al*., 2005). Adventitious shoot formation in cotyledon occurred mainly until 10 WAP after the explants were put into the media. After 10 WAP some shoot emerged, however they were abnormal, and after a while the shoot stopped growing and finally dried out.

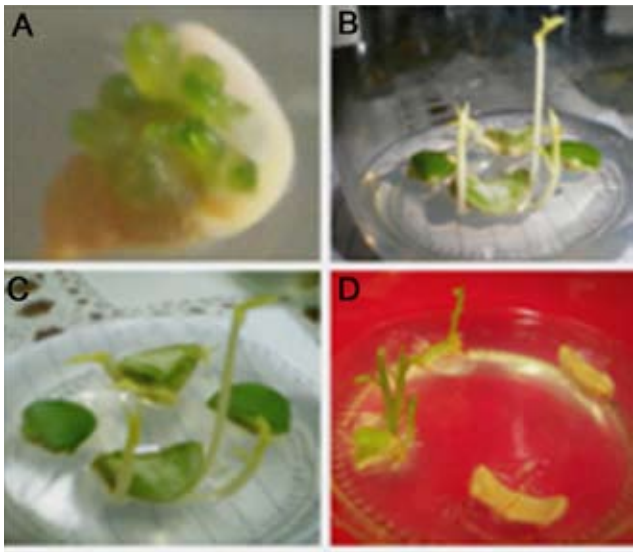

Figure 1. Adventitious shoot formation from cotyledon explants of Pummelo cv Cikoneng. (A) shoot initiation, nodules grew directly from cotyledon, (B) white shoot emerged from dark incubation; (C) under the light shoot leaf started to green; (D) green cotyledon with multiple shoots and non-responsive cotyledon; shrinked and turned yellow

Adventitious shoots initiation in epicotyl was preceeded by callus formation around cutting position of epicotyl segments (Figure 2). The adventitious shoots were formed from the callus. Similar pathway was reported by Saini dan Jaiwal (2002) in epicotyl culture of *Vigna mungo* where adventitious shoots were formed from callus (indirect organogenesis). According to Filho (2001) callus type formed at the epicotyl segments was type I callus which was not friable and could differentiate directly to form special organ like shoot or leaf. However, plants obtained from disorganized tissues such as callus may not be true to type due to somaclonal variation (Singh *et al*., 2010). These variants may provide advantageous characteristics such as pest and disease resistant or even different fruit shape, color, and juiciness, but maintenance of genotypic and phenotypic identity is an indispensable requirement for mass propagation (Paudyal and Haq, 2000). Thus, plants obtained from this pathway should not be used in *in vitro* conservation. The time needed to form shoots in epicotyl ranged between 6-8 WAP. Similar to cotyledon explants, the earliest adventitious shoots were formed in media 1 and media 3. Appearance of adventitious shoots formed from epicotyl was different from that formed from cotyledon. Adventitious shoots from epicotyls looked weak, transparent or vitrous due to hyperhydricity and their growth was slower (Figure 2, right) than that obtained from cotyledons (Figure 1, upper right, bottom). The special conditions during *in vitro* culture often results in the formation of plantlets of abnormal morphology, anatomy and physiology (Hazarika, 2006).

Not all cultured explants yielded adventitious shoots. The number of adventitious shoots obtained from each segment of cotyledon and epicotyl ranged between 1-5 shoots. Percentage of explants forming adventitious shoots

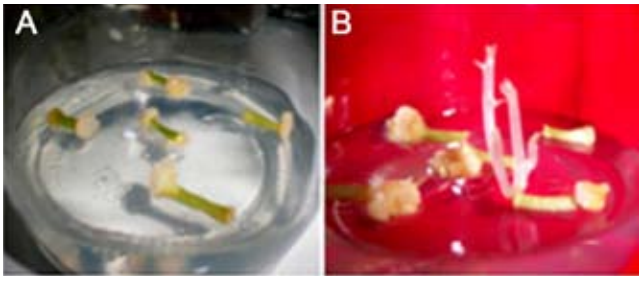

Figure 2. Formation of adventitious shoots from epicotyl of Pummelo cv. Cikoneng. (A) callus formed in two ends of epicotyl segments, (B) shoots showing vitrous appearance

at the end of observation of 13 weeks after planting (WAP) in all media is presented in Table 1. The highest percentage of explant forming adventitious shoots was reached by cotyledon in media 1 (38.8%), eventhough it was similar to cotyledon in media 3. The lowest percentage of explant forming adventitious shoots was reached by epicotyl in media 4 and media 5 (4.0%).

Interaction of media and type of explant affected the number of shoot (Figure 3). Number of shoot in cotyledon in media 1 and 3 increased only in the first four weeks after subcultured (Figure 3). The highest number of shoot (58 shoots) was obtain in media 3 followed by 50 shoots in media 1 (Figure 3A). In epicotyl explants, the number of shoot formed was lower than that in cotyledon. Epicotyl explant in media 3 only gave 15 shoots, the highest among other media treatment (Figure 3B). The shoots emerged from epicotyl also grew slower. After 12 WAP there was no shoot emerged.

Percentage of adventitious shoot to number of explant is presented in Table 1. This showed the efficiency of shoot formation per cultured explant. The number of shoot was counted from both normal and abnormal shoots. The highest efficiency was obtained on cotyledon cultured in media 3 (72.5%), while the lowest was obtained on epicotyl in media 5 (5.0%). The highest percentage of shoot to number of explant in epicotyl explant was also obtained in media 3, i.e. 15% (Table 1). This might be due to the explant size, where larger explant size such as cotyledon (Figure 1) gave more energy source for and faster adventitious shoot initiation than segmented epicotyl explant (Figure 2). The research in bottle gourd (*Lagenaria siceraria*) using cotyledon explants gave similar results, where synergistic effect of Kinetin and Benzyl Adenine (BA) enhanced the shoot regeneration efficiency compared with BA and Kinetin used separately (Saha *et al*., 2007).

A summary of variance analysis in adventitious shoot growth and rooting at the end of observation of 12 weeks after subcultured (WAS) is presented in Table 2. Treatment of media, type of explants and their interactions significantly influenced shoot height, leaf number, and rooting. Effect of interaction of explant type and media on shoot height is presented in Table 3. In general, the height of shoot obtain from cotyledon was higher than those obtained from epicotyl. Growth of shoots obtained from epicotyl was slower than that obtained from cotyledon. Table 3 showed that treatment media 1 + cotyledon achieved the highest shoot height, but it was not significant to treatment media 3 + cotyledon. However, those interactions of cotyledon and media 1 or media 3 were significantly different to other

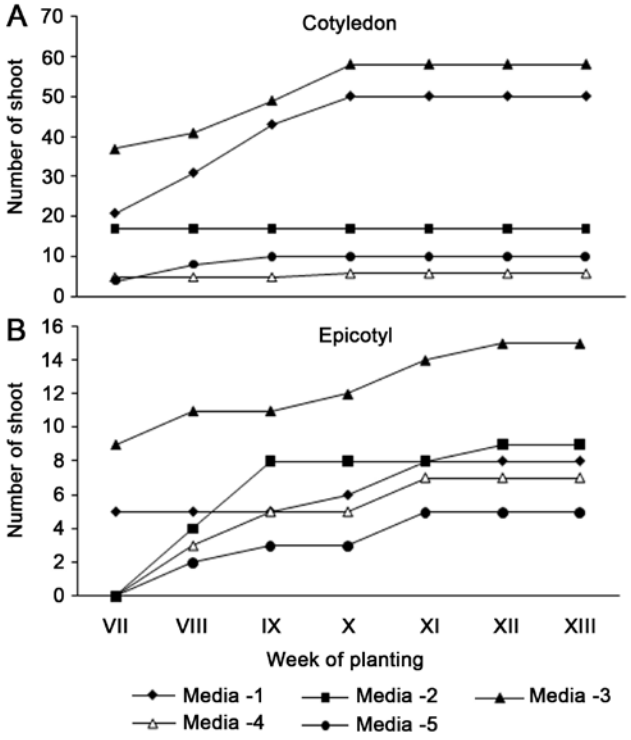

Figure 3. Interaction of media and explant type on number of adventitious shoots obtained from (A) cotyledon and (B) epicotyl of Pummelo cv. Cikoneng from 7-13 WAP; Media 1 = MS + 1.0 mg BAP L^{-1} + 0.5 mg Kn L^{-1} + 0.5 mg NAA L^{-1}; 2 = MS + 2.0 mg BAP L^{-1} + 0.5 mg Kn L^{-1} + 0.5 mg NAA L^{-1}; 3 = MS + 1.0 mg BAP L^{-1} + 1.0 mg Kn L^{-1} + 0.5 mg NAA L^{-1}; 4 = MS + 2.0 mg BAP L^{-1} + 0.5 mg Kn L^{-1} + 1.0 mg NAA L^{-1}; 5 = MS + 2.0 mg BAP L^{-1} + 1.0 mg Kn L^{-1} + 1.0 mg NAA L^{-1}; Kn = Kinetin

Table 1. Percentage of explant forming adventitious shoots and efficiency of shoot formation per cultured explant of Pummelo cv. Cikoneng

Explant type	Media 1	Media 2	Media 3	Media 4	Media 5
		…… Explant forming adventitious shoots (%) ……			
Cotyledon	38.8	8.8	36.3	7.5	8.8
	7.0	8.0	11.0	4.0	4.0
		……..Efficiency of shoot formation (%)……			
Epicotyl	62.5	21.3	72.5	7.5	12.5
	8.0	9.0	15.0	7.0	5.0

Note: Media 1 = MS + 1.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; 2 = MS + 2.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; 3 = MS + 1.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; 4 = MS + 2.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 1.0 mg NAA L^{-1}; 5 = MS + 2.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 1.0 mg NAA L^{-1}

Table 2. Summary of analysis of variance on media and explant treatments and their interactions in adventitious shoot growth and rooting at 12 WAS

Variable	Treatment		
	Media	Explant	Media x explant
Shoot height[a]	**	**	**
Leaf number[b]	**	**	**
Root number[a]	**	**	**

Note: WAS = weeks after subculture; ** = highly significant (α = 1%); a = Data was transformed with $\sqrt{(x+0.5)}$; b = Data were transformed with $\sqrt{(x+1)}$

treatments. At 12 WAS, shoot height from cotyledon could reach 0.9 cm to 2.0 cm while those from epicotyl could reach only 0.8 cm to 1.1 cm. Height increase was occurred along with every leaf grew on the plantlet.

Observation on leaf number of shoot obtained from cotyledon and epicotyl was conducted 1 week after the shoots were subcultured. Effect of interaction of explant type and media on number of shoot leaf is presented in Table 3. In general, number of leaf was ranged from 1 to 2 leaves. Leaf growth was slow. Figure 4 showed the average number of leaf up to 12 WAS. The leaf number can be used as indicator for the number of internode. The low number of leaf influenced plant height. The lesser the leaf number the lower the shoot height (Table 3). Leaf in cotyledon derived-shoot in media 1 and 3 was increased until 12 WAS, but cotyledon derived-shoot in media 2, 4, 5 did not show any leaf increase after 4 WAS. In 12 WAS, the highest number of leaf was achieved by shoots obtained from cotyledon cultured in media 1 and media 3 (Table 3). Leaf increase in epicotyl derived-shoot in media 1 occured after 4 WAS and no further increase until 12 WAS. Epicotyl derived-shoot in media 2, 3, 4, 5 did not show any increase of leaf number after 1 WAS (Figure 4).

Only normal shoot gave roots (Figure 5). Vitrous or hyperhydric shoots obtained from epicotyl could not form root. Root did not appear until 2 WAS. After 3 WAS root formed on normal shoots planted in rooting media containing active charcoal (Figure 5). The earliest root obtained from cotyledon and epicotyl-derived shoots was from media 1. At the end of observation, shoots obtained from cotyledon cultured in media 1 or media 3 gave the highest number of root (Table 3).

The difference of media 1 and media 3 is in kinetin concentration. Formulation of media 1 was MS + 1.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}, while of media 3 was MS + 1.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}. Media 1 (0.5 mg L^{-1} Kinetin) contained less kinetin than media 3 (1 mg L^{-1} Kinetin). Since the effect on shoot height and number of roots derived from cotyledon were not significant in both media 1 dan 3 (Table 3), therefore the use of media 1 was more efficient in shoot growth of *Citrus maxima* L. This result is supported by previous reports in various Citrus species. Al-Khayri and Al-Bahrany (2001) conducted research on propagation of *Citrus aurantifolia* and reported that MS + 0.5 mg Kinetin L^{-1} +1.0 mg BAP L^{-1} gave the best shoot growth. In *Citrus reticulata* Blanco and *Citrus jambhiri* Lush media MS + 2.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 1.0 mg NAA L^{-1} also gave the best result (Ramkrishna *et al*., 2005). The protocol is consistent and reproducible, and the results showed a high efficiency level in the regeneration of whole plants. In another experiment using cotyledon as explant and media MS +1.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}, two other cultivars of pummelo, i.e. Nambangan and Srinyonya were successfully propagated and stored as mother stock (Figure 6).

Table 3. Interaction of explant type and media on adventitious shoot height, number of leaf, and number of root of Pummelo cv. Cikoneng at 12 WAS

Explant type	Media 1	Media 2	Media 3	Media 4	Media 5
		.....Shoot height (cm)			
Cotyledon	2.0a	1.3b	1.7a	0.9c	1.1bc
Epicotyl	0.8c	0.8c	1.0bc	0.8c	0.8d
		.....Number of leaf			
Cotyledon	1.9a	1.5bc	1.9a	1.4c	1.6b
Epicotyl	1.4c	1.3c	1.4c	1.3c	1.3c
		……Number of root……			
Cotyledon	1.1a	0.8b	1.1a	0.8b	0.9b
Epicotyl	0.7c	0.7c	0.8bc	0.7c	0.7c

Note: Media 1 = MS + 1.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; 2 = MS + 2.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; 3 = MS + 1.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; 4 = MS + 2.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 1.0 mg NAA L^{-1}; 5 = MS + 2.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 1.0 mg NAA L^{-1}; numbers followed by same letter within the same column are not significantly different according to LSD at α = 5%

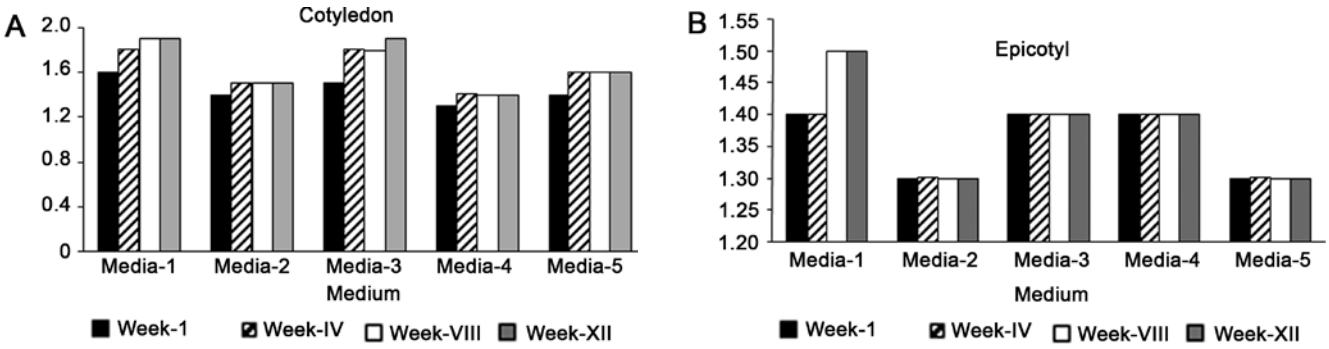

Figure 4. Interaction of explant type and media on shoot leaf number of Pummelo cv.Cikoneng at 1, 4, 8, and 12 WAS. (A) Shoot leaf number in cotyledon-derived shoot and (B) epicotyl-derived shoot in media 1 = MS + 1.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; 2 = MS + 2.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; 3 = MS + 1.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1}; 4 = MS + 2.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 1.0 mg NAA L^{-1}; 5 = MS + 2.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 1.0 mg NAA L^{-1}

Figure 5. Growth of root from normal adventitious shoots of pummelo cv. Cikoneng subcultured in rooting media containing active charcoal

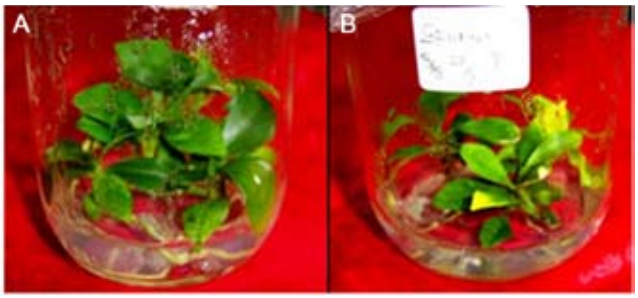

Figure 6. *In vitro* collection of Nambangan (A) and Srinyonya (B) resulted from cotyledon-derived shoots induced by media MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} Kinetin + 0.5 mg L^{-1} NAA

CONCLUSION

Treatment of cotyledon explant cultured in media 1, i.e. MS + 1.0 mg BAP L^{-1} + 0.5 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1} and media 3, i.e MS + 1.0 mg BAP L^{-1} + 1.0 mg Kinetin L^{-1} + 0.5 mg NAA L^{-1} gave the highest percentage of explants forming adventitious shoots (38.7% and 36.3%, respectively), efficiency of shoot formation (62.5 dan 72.5%, respectively), leaf number (average of 1.9 leaf $shoot^{-1}$), and root number (average of 1.1 root $shoot^{-1}$). Those combinations of treatments were the fastest in shoot initiation (4 to 5 WAP). However, it is suggested that cotyledon and media 1 which is less in Kinetin is used for pummelo propagation due to non-significant effect on shoot growth, i.e. shoot height, number of leaf, and root.

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